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Generation Cycle of Mesenchymal Cells of Palatal Processes of Cleft Palate Wistar Albino Rat Fetuses

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GENERATION CYCLE OF MESENCHYMAL CELLS OF PALATAL PROCESSES OF CLEFT PALATE WISTAR ALBINO RAT FETUSES

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INTRODUCTION

Clefts of the lip and palate are among the most common congenital anomalies in humans. Statistics vary depending on the country, race, sex and type of cleft, but on the average, the incidence of cleft of the lip with or without cleft of the palate is in the range of one in 900 births. It is a severe handicapping anomaly, affecting the esthetics and oral function of the affected person, frequently leading to psychological problems. Consequently, numerous investigations have been conducted to study the pathogenesis of this anomaly in human abortus material as in laboratory animals.

The normal secondary palate development can be described as occurring in four distinct morphologic stages. They are basically the same in humans and animals and are described as follows: 1) in the early stage of embryonic palatal development, the palatal processes are growing in a vertical direction with the tongue positioned between them; 2) transposition of the vertically positioned processes into a horizontal orientation, superior to the lowered tongue; 3) growth of the horizontal processes towards each other until they approximate in the mid-line; and 4) breakdown of the epithelial lining between the processes and fusion with each other. These major steps have been well documented by various investigators. However, the mechanism of each individual step is not well understood. Disturbance in any of these stages could result in non-fusion of palatal processes (cleft palate).

The major part of the past work was directed towards toxicological and gross morphological changes in the normal and abnormal development of the secondary palate. Recently more attention has been devoted to basic cellular and biochemical changes and their role in the spatial change, growth, and ultimate fusion of the palatal processes. Several investigators have reported that various cleft palate producing teratogens affect cell proliferation during the morphogenesis of the secondary palate. These studies will be reviewed in the literature review section of this paper. In addition, cell proliferation has been suggested to play a direct role in the horizontal movement of the palatal processes prior to their approximation and fusion.

The purpose of this investigation is to determine the generation cycle of the mesenchymal cells of the palatal processes of rat fetuses from vitamin A treated mothers on days 15 to 16 of gestation during which horizontalization of palatal processes occurs. The generation cycle of the mesenchymal cells will be evaluated on the basis of the number of proliferating cells and the nuclear cycle time.

Hypervitaminosis A will be used as a teratogen to produce cleft palate.

LITERATURE REVIEW

Normal secondary palate morphogenesis can be divided into four distinct sequences of events:

1. Growth of the vertical palatal processes
2. Transposition of the vertically directed palatal processes into a horizontal position
3. Growth of the horizontal palatal processes toward each other
4. Fusion of the palatal processes

The horizontal movement of the vertically positioned palatal processes is a major area of disagreement in the literature. As early as 1920, Shaeffer reported that differential growth of the palatal processes resulted in this positional change. Regression at the inferior border of the vertically positioned palatal processes with concomitant outgrowth of the medial border was proposed by Pons-Tortella (1937). This theory was later supported by Orban (1957) and Coleman (1965).

Walker and Fraser (1956) first proposed that there was an internal force in the palatal processes which gradually increased in strength until it drove them into a horizontal position. Larsson (1960) and Walker (1961) postulated that a gradual buildup of acid mucopolysaccharides in the ground substances of the palatal processes produced the internal force necessary to reorient the processes. Larsson (1962a) showed a diminished content of acid mucopolysaccharides in palatal tissues of embryos with cortisone induced cleft palate. However, Nanda (1969, 1970a) stated that the ^{35}S-sulfate uptake in the different groups of clefts induced by cortisone, vitamin A, and a combination of these two agents showed no positive correlation between the occurrence of cleft

palate and disturbed mucopolysaccharide metabolism.

Walker (1961, 1967) used hyaluronidase to inhibit the mucopolysaccharides present in the palatal processes. His experiments failed to disrupt the movement of the palatal processes, which suggested that another mechanism may be responsible for their convergence. Anderson and Matthiessen (1967) suggested vascularity as another causative factor in the development of the secondary palate. They found that vascular development in the palatal processes and the adjacent anatomical areas takes place concomitant with the transposition of the palatal processes.

The tongue has been reported to play a role in the horizontalization of the palatal processes (Lazzaro, 1940; Walker, 1969). The change in relationship between the tongue and the palatal processes is crucial for the palatal fusion. During this change the tongue shifts downward and the palatal processes become horizontal. The cause and effect relationship of this change is still not determined. Several observations have been reported in this regard by several investigators. It has been suggested that the tongue drops down as a result of oral reflexes (Humphrey, 1969), growth of the mandible in a downward direction (Nanda, 1969) and due to the change in the cranial base flexure (Harris, 1964).

The tongue has also been discussed as a possible force for providing elevation of the palatal processes, although evidence has not been presented to support the theory. Moriarty et al (1963) have shown that elevation may occur without the presence of the tongue.

The last phase of the palatal fusion, consists of formation of the wall of the epithelium after approximation of the palatal process, fusion of the approximated epithelial cells and their subsequent degeneration, and coalescence of the underlying mesenchymal cells. These events have been studied in detail, utilizing electron microscope and *in vitro* techniques.

Pourtois (1968) described the formation of a "zone of stickiness" by the cell layers differentiating at the edge of the processes which eventually fused, leading to the formation of a laminated wall of epithelium between the processes.

DeAngelis and Nalbandian (1968) found that the palatal processes of mice and rats exhibited an irregular outer epithelial plasmalemma which became more regular just prior to contact. Desmosomes were found at the contacting surfaces and appeared to bind the two new processes together until the mesenchymal union of the processes.

The desintegration of the laminated epithelial wall after the epithelial fusion of the palatal processes has been investigated with the aid of the electron microscope. Signs of autolysis (Farbman, 1968), numerous types of lysosomes in the cells at the time of desintegration (Mato et al. 1965) and the presence of hydrolytic enzymes such as acid phosphatase within the epithelial layers (Angelici and Pourtois, 1968) have been attributed to the breakdown of the laminated epithelial cell wall. Angelici and Pourtois (1968) stated that the absence of acid phosphatase activity in the epithelial wall could eventually lead to the reopening of the seam which may result in the formation of a cleft.

Shapiro and Sweeney (1969) investigated the programmed cell death theory of the epithelial cells during fusion. They found ultrastructural evidence of decreased respiration in these cells. Hudson and Shapiro (1973) found a decline in mitotic activity of the palatal tip epithelium (presumptive zone of fusion) supporting the concept of programmed cell death.

Of the teratological agents used to cause and study cleft palate formation in laboratory animals, hypervitaminosis A is one of the most widely used (Cohlan, 1954; Kochhar and Johnson, 1965; Marin-Padilla, 1966; Myers et al.1967; Kochhar 1968; Nanda, 1969, 1971, 1974). However, explanations differ as to how maternal hypervitaminosis A leads to fetal cleft palate. Woollam and Millen (1958, 1960) postulated an effect via an alteration of the maternal carbohydrate metabolism. Giroud et al. (1957) proposed a direct effect of hypervitaminosis A on the fetuses, as they found an increase of vitamin A in the fetuses after its administration to pregnant rats. Others have reported structural changes in the craniofacial complex of fetuses from vitamin A treated rats which might directly or indirectly influence fusion of the palatal processes. Some of these structural changes include: a short mandible and maxilla (Cohlan, 1954), precocious cartilage formation in the maxillo-mandibular area (Steffek et al. 1966; Nanda 1970), and alterations in the metabolism of the palatal processes as evidenced by their increased uptake of S^{35}sulfate (Kochhar and Johnson, 1965).

Kochhar (1968) and Nanda (1969, 1971) showed a decrease in cell proliferation in fetuses from vitamin A treated rats. They used tritiated thymidine labeling as indicator of DNA synthesis and found a

decrease in labeling index when compared to controls. Similarly, Mott et al. (1969), and Jelinek and Dostal (1974) used cortisone as the teratogenic agent and found suppression of the proliferation of cells in the palatal processes.

Nanda, (1975) demonstrated that the effects of vitamin A on the potentiality of rat palatal processes to fuse in vitro was reversible. Nanda and Romeo (1975, 1978) found significant differences in tritiated thymidine labeling on days 14 and 15 of gestation between fetuses treated with vitamin A when compared to normal but failed to find a significant difference in the labeling on days 16 and 17 of gestation, indicating a 'catch-up' phenomena.

The investigations reporting on the effect of hypervitaminosis A in other tissues are numerous and varied. Cohlan (1953) was the first to describe the effects of vitamin A on embryonic development. He found brain deformities and ocular abnormalities among other defects (1954).

Morriss(1975)and Hassell et al. (1977) reported abnormal neural crest cell development and migration in their studies with hypervitaminosis A. In a retrospective study, Gal et al. (1968) attributed some CNS defects in humans to an increased amount of vitamin A in the blood.

Aydelotte (1963 a,b) found an interference with mitosis in in vitro studies of chick tracheal, esophageal and corneal epithelium. On the other hand, Lasnitzki (1954) reported an increased mitotic rate and growth rate effect of hypervitaminosis A on chick heart fibroblast in vitro.

Kwasigroch and Kochhar (1975) demonstrated impaired cell motility in cultured limb bud mesenchyme in the presence of vitamin A. Abnormal cell migration may result from factors acting directly on the cells, or from chemical alterations in the matrix through which the cells migrate, Morriss (1975). Morriss, (1973) and Morriss and Steel, (1974) reported that hypervitaminosis A causes expansion of cell membranes, blebbing of cells and ultimate death of these cells. They also reported that the cell matrix expanded in volume, particularly in the mesenchymal layer.

Fell et al. (1952) found skeletal changes and growth arrest in embryonic limb bones in vitro, with high doses of vitamin A. Misra, (1968) found a marked decrease in food consumption in hypervitaminosis A treated rats.

Barnet and Dzabo (1973) found a decrease in the epithelial keratinization in cultures with vitamin A. Leelaprute et al. (1973) reported an increase in bone radiolucency and ectopic soft tissue calcification in their in vivo studies with hypervitaminosis A in rats.

The present investigation was undertaken to study the role of cell proliferation in the growth and development of the secondary rat palate with special emphasis on the horizontalization stage of their development.

The generation cycle of mesenchymal cells of palatal processes in fetuses from hypervitaminosis A treated mothers will be studied and will be compared to the normal data.

MATERIALS AND METHODS

Sixty (60) nulliparous female Wistar albino rats weighing approximately 250 gms were used in this study. They were housed in a constant temperature room (72°F) with controlled light and dark cycles to minimize diurnal variation (light cycle from 6:00 A.M. to 6:00 P.M.). Water and food (Purina Lab Chow) were supplied ad libitum. All rats were purchased from the same commercial breeding laboratory (Charles River, Massachusetts) and allowed to remain for one week in the vivarium prior to mating, to allow for acclimation. The rats were mated during the dark cycle. Each female rat was placed with a male Wistar rat at approximately 12 midnight and removed between 7:00 - 8:00 A.M.. Vaginal smears were performed and if found positive, it was recorded as day 0 of gestation. Each pregnant female rat was placed in an individual cage and the weight recorded.

All pregnant rats received 50,000 I.U. of vitamin A (1.0 ml Aquasol A Drops - USV Pharmaceutical Corp.) once a day via stomach tube on days 9, 10, 11, and 12 of gestation. The vitamin A was administered as close to 8:00 A.M. as possible.

At 8:00 A.M. on day 15 of gestation, 50 of the pregnant rats were given an intraperitoneal injection of tritiated thymidine. (^{3}HTdr - obtained from New England Nuclear, sterile aqueous solution in combi-vial with a specific activity of 6.7 Ci/mmol. The dose was 250 µCi/Kg of rat body weight.

Each rat was randomly assigned to a sacrifice schedule. The schedule was as follows: Two rats were sacrificed at 0.5 hour and

thereafter at hourly intervals for a period of 24 hours. The rats were sacrificed by placing them in a glass desiccator with ether until anesthesia was achieved. The chest cavity was then opened and the aortic artery severed. They were allowed to exsanguinate to death. The abdomen was opened and the two uterine horns exposed. The number of live, dead and resorbed fetuses was recorded. The wet weight of each fetus was obtained on a Sartorious top loading balance.

The heads of four fetuses from each rat were fixed in Bouin's solution for a period of 24-48 hours. The remainder of the fetuses were placed in 10% unbuffered formalin for later macroscopic examination.

After the period of fixation in Bouin's, the heads were washed in 50% ETOH and processed in a Shandon-Elliot automatic processor. They were embedded in parafin and 5 µm sections cut in a frontal plane. The sections were dipped in Kodak NTB-2 liquid emulsion to obtain a thin film coating over the tissue sections. They were exposed in a light-tight container at 4°C for a period of 26 days. The sections were then developed with Kodak D-19 developer at 18°C and stained with hematoxylin-eosin.

The total number of serial sections cut in the frontal plane were counted from the first appearance of the secondary palatal processes to the last section, where the palate was no longer visible. The total number of sections was divided into three equal parts in order to determine the middle region of the palate. A total number of 1000 mesenchymal cells of the palatal process was counted from the middle region of the secondary palate. A Zeiss photomicroscope with an eyepiece grid was utilized. All sections were viewed under oil immer-

sion by the same investigator. At the time of counting, the investigator did not know the hour of sacrifice in order to eliminate bias.

The number of labelled and unlabelled cells, labelled and unlabelled mitosis were recorded with the aid of a Clay-Adams laboratory counter. A minimum of 4 grains per nucleus was used to consider a cell labelled. The average cell density in the palatal processes was also recorded. This was taken as the number of cells per area of the eyepiece grid. A total of 10 grid spaces were counted and averaged for each fetus. Random slides were recounted to determine the reliability of the counts. A second investigator was also asked to count some slides to compare results.

The remaining 10 vitamin A treated pregnant rats were sacrificed on day 20 of gestation in the same manner as described previously. The fetuses were removed, examined and placed in 10% unbuffered formalin. These rats were used to determine the percentage of cleft palates produced in the fetuses by the dose of vitamin A mentioned above.

At the time of sacrifice of the pregnant rats, the following observations were made:

1. Mothers' body weight
2. Number of live, dead and resorbed fetuses
3. Wet weight of live fetuses
4. Fetal gross malformations
    a) Malformations of the head
    b) Malformations of the limbs
    c) Agnathia or micrognathia
    d) Malformations of tails

e) Syndactyly

f) Cleft lip and/or palate

The corresponding control data for this investigation was obtained from the study of Romeo and Nanda (1978). This study was performed in the same laboratory under identical conditions. All their slides were examined again to determine variation between investigators, and the difference in recordings was found to be no greater than 10%.

RESULTS

One of the aims of the present study was to administer a dose of vitamin A to pregnant rats which would produce a maximum number of fetuses with cleft palate. A preliminary study was performed to obtain a desirable dosage of vitamin A. A dose of 60,000 I.U. was given to pregnant rats once a day from day 9-12 of gestation. This dosage produced 34-100% resorptions. The dosage was then reduced to 50,000 I.U., which reduced the resorptions to 20% and increased the percentage of live fetuses with cleft palates to 94% (Fig. 1).

Macroscopic findings: No attempt was made in the present investigation to record systematically the number of anomalies other than the clefts of the palate. The most common abnormalities observed other than clefts of the palate were steep frontal region of the face and head, open eyes and exophthalmus. Other malformations exhibited were hydrocephalus, micrognathia, retrognathia, malpositioned ears and short limbs. One fetus exhibited a cleft of the mandible (Fig. 2). The average wet weight of the 15-16 day old fetus was 0.30 gms. Most of the variation in weight was dependent on whether the time of sacrifice was closer to 15 days or 16 days of gestation.

Microscopic findings: Microscopic examination was performed only on fetuses used for the autoradiographic studies. The palatal processes were oriented in a vertical direction with the tongue positioned between them. They were small and often stubby. Tooth buds were often missing unilaterally or bilaterally. The nasal septum was bulbous and short in a vertical direction. The eyes were frequently bulging and open. The palatal processes were composed of loose and undifferentiated mesenchymal cells. One or two layers of cuboidal epithelial cells

covered the processes (Figs. 3, 4). The average cell density of the palatal processes did not vary between the different hour fetuses.

Autoradiographic findings: Different exposure times were tried in the preliminary study to determine an optimum exposure time. The best results were achieved after an exposure of 26 days at 4°C. (Fig. 5).

The percent labelled cells at different sacrifice times is shown in (Fig. 6). The mean percentage of labelled cells at 1 hour was 22.3% which increased to 57% two hours after the ^{3}HTdr injection. It then decreased slightly to 48.4% at 4 hours and again increased to 54% at 6 hours. A marked drop to 35.9% was noted at 7 hours followed by a steady increase to 55.7% at 9 hours. Between 9 hours and 24 hours it fluctuated $\pm$ 12% reaching a high of 56% at 12 hours and low of 44% at 22 hours (Fig. 6).

Figure 7 shows the mean percent labelled mitoses at different hours after the ^{3}HTdr injection. At 1/2 hour 18.6% of the observed mitoses were labelled. The percent of labelled mitoses increased steadily until five hours, at which time 58% were labelled. It then went down to 51.6% at 6 hours and again fluctuated up to 60% at 9 hours. A decrease was then observed until it dropped to 36.5% at 11 hours. The period between 11 hours to 19 hours demonstrated a fluctuation with a high of 45.8% at 12 hours and low of 31% at 19 hours. A second peak was noted at 21 hours with a mean of 63.2% which decreased to 28% at 24 hours (Fig. 7).

A sample number of cells were examined for number of grains per cell at different hours. In the vitamin A group (contrary to the control group), (Romeo and Nanda, 1978) no appreciable decrease in the number of grains was noted in the fetuses at all hours.

Appendix 1 illustrates the number of cells in mitoses per 1000 cells for each hour of sacrifice. Appendix 2 represents the number of labelled mitoses per 1000 cells for each hour of sacrifice.

DISCUSSION

The macroscopic findings of this study are consistent with previous studies reported in the literature. Cohlan (1952, 1954) was the first to discuss the effects of vitamin A on embryonic development and ever since, the literature has been abundant on its teratogenic effects on different animals. Anencephaly, exencephaly, anophthalmia, microphthalmia, spina bifida, retrognathia, micrognathia, syndactyly, limb deformities as well as cleft palate has been reported in rat fetuses of mothers treated with hypervitaminosis A (Cohlan, 1954; Giroud and Martinet, 1956; Nanda, 1969; Morriss 1972; Nanda and Romeo, 1977b), in mice (Kalter, 1960; Kalter and Warkany 1961) and in hamster (Marin-Padilla and Ferm, 1965). The exact nature in which hypervitaminosis A causes all these malformations is still not clear. Some of the possible explanations are the inhibitory effect of vitamin A on neural crest cell differentiation and cell migration (Langman and Welch, 1967; Morriss, 1975; Hassell et al. 1977), skeletal interference (Fell and Mellanby, 1952; Leelaprote et al. 1973), disturbance of the epithelial-mesenchyme interaction (Kochhar, 1968) and overall growth disturbance (Fell and Mellanby, 1952; Kalter, 1960).

The detailed study of the gross morphologic malformations is beyond the scope of this investigation. This study was designed to investigate the role played by hypervitaminosis A treated pregnant rats on the cell proliferation of the palatal processes of their fetuses.

Previous studies strongly suggest that cell proliferation plays a major role in palate morphogenesis. A decrease in cell proliferation could result in unfused palatal processes as a result of stunted growth

of the processes (Kochhar and Johnson, 1965; Abramovich and Devoto, 1967; Lotosch, 1968; Nanda, 1969), interference with horizontalization (Lassaro, 1940; Orban, 1957; Andersen and Matthiensen, 1968; Kochhar, 1968; Nanda and Romeo, 1975), or a combination of both.

No previous investigation has studied the cell cycle of mesenchymal cells in palatal processes of animals with hypervitaminosis A induced cleft palates, although an increased cell cycle has been reported in other tissues of hypervitaminosis A treated animals (Langman and Welch, 1966, 1967; Kochhar, 1968).

The cell cycle is divided into four phases: 1) the DNA synthesis period, S phase, during which the amount of DNA in a cell is doubled in preparation for the division into two daughter cells (this is the phase in which the labelled thymidine is incorporated); 2) the G_2 phase follows the S phase (during this phase the cell contains a double complement of DNA); 3) the mitotic phase, M, follows G_2. (In this phase the cell divides into two daughter cells, each with a single complement of DNA); 4) the G_1 or interphase also called the resting phase, follows mitosis. After the division, the daughter cells could enter again into the cell cycle at S phase or go into G_0 phase in which they are no longer part of the proliferating pool.

An investigation of the parameters of cell cycle of the nature of this one makes certain assumptions. It must be assumed that we are dealing with a steady state situation in a homogeneous population which is growing exponentially and in which a random distribution of cells throughout the different phases of the cell cycle exists. It must also assume that cell death and cell migration are negligible.

The data shown in Fig. 7 indicates that in a sample of the cell population studied, the DNA synthesis occurs in a cyclic pattern. Estimation of the G_2 phase can be done from the first appearance of labelled mitosis. A G_2 of 1/2 hour was found for the mesenchymal cells in the present study. This is consistent with the value of G_2 found for the mesenchymal cells of normal palatal processes reported by Romeo and Nanda (1978). Although they report a value for G_2 of 1 hour, they recognize the possibility of a shorter value due to the fact that they did not include time period earlier than 1 hour after the ^{3}HTdr injection.

The S phase is estimated by measuring the interval between the 0.5 point on the ascending leg of the first mitotic wave to the 0.5 point on the descending leg of the same mitotic period (Wimber, 1963; Cleaver, 1967). The S phase was estimated to be from 8-9 hours which was noted to be 4 hours longer than the control group (Romeo and Nanda, 1978). This is in agreement with the study of Langman and Welch (1967) in which they found that the cell cycle in the neuroepithelial cells of mouse fetuses from mothers treated with hypervitaminosis A had a longer DNA synthetic phase. An interference with DNA synthesis on the mesenchymal cells of palatal processes from fetuses of hypervitaminosis A treated mothers was also reported by Kochhar (1968) and Nanda (1971). It must be noted here that these studies only reported on the labelling index of the cells of the palatal processes from 1 to 5 hours after the injection of ^{3}HTdr. Furthermore, the embryonic period studied in these investigations does not exactly correspond with that of the present study.

A mitotic phase of 3 hours was calculated from Figure 7. It was found to be 1.5 hours longer than the normal mitotic phase reported by Nanda and Romeo (1978). This finding supports the results of Langman and Welch (1967) that the vitamin A interferes with mitoses. On the other hand the present results do not support the findings of Lasnitzki (1954). He found in an in vitro study on the effect of excess vitamin A on chick heart fibroblasts, an increased mitotic rate. He concluded that this could be a result of lack of vitamin A in his control as opposed to the effect of the vitamin in his experimental group.

The total generation cycle time, the interval between the peaks of the two mitotic waves, was estimated to be approximately 15 hours. This is 3 hours, shorter than the normal cycle reported by Romeo and Nanda (1978). This difference could be attributed to the peak of the second wave of mitoses being determined from the graph by only one point at 21 hours. A possibility exists that this increase does not represent a second wave or cycle, but a continuation of the plateau observed in the graph after 9 hours.

An analysis of Figure 7 also suggests that cell proliferation in the vitamin A group was arrested at the S to M transition phase of the generation cycle. The fact that the percent labelled mitoses never reached a level as high as the normal group would seem to support this possibility. Furthermore, cells which are arrested in the S or pre-S phase at the time of ^{3}HTdr injection, will not incorporate the labelled thymidine, therefore, decreasing the percent of labelled mitoses. Failure of the percent labelled mitoses to reach 100% in both experimental and normal animals (Fig. 7) could be attributed to the possibility of more than one cell population composing the mesenchymal tissue of the palatal processes with differing

nuclear cycle times.

Assuming that all cells are proliferating, the fraction of labelled cells (FLC) could be predicted by the formula FLC = Ts/Tc, where Ts = length of the S phase and Tc = the length of the total generation cycle. Using the values arrived from the data in this study a predicted FLC in vitamin A treated animals would be 53% (FLC = 8/15 = 0.53).

Examination of Figure 6 at the peak of the first wave of the vitamin A group, which is approximately 6 hours, shows 53% of the cells of the experimental group are labelled. This would suggest that shortly after the injection of ^{3}HTdr, all cells are proliferating. If all cells continue to proliferate, it would be predicted that the fraction of labelled cells would double at the time of the second wave. However, Figure 6 shows that the fraction of labelled cells in the vitamin A treated group has remained approximately the same.

Applying the same formula to the normal group studied by Romeo and Nanda (1978), 22% of the cells would be labelled at the time of the first wave (FLC=Ts/Tc=4/8=0.22). An analysis of Figure 6 shows 19% of the cells were labelled. Further examination of the graph indicates that at the time of the second wave, the percent of labelled cells in the normal group has doubled as expected. This suggests that all the cells in the palatal processes from the normal group are proliferating, whereas they have not continued to proliferate in the experimental group.

With each cell division, the amount of labelled thymidine incorporated in the DNA of the nucleus will be diluted. This would be evidenced by a decrease in grains per nucleus as the cell cycle progresses, providing no additional ^{3}HTdr is available to the cells for incorporation. The fact

that the average number of grains per nucleus in the vitamin A group does not reduce significantly, gives support to the possibility of a cell cycle arrest.

This study primarily attempted to investigate the role of cell proliferation in the production of cleft palate in fetuses from mothers treated with hypervitaminosis A at the time when the palatal processes are normally becoming horizontal. At the present state of knowledge, it is probably safe to assume that more than one mechanism is involved in the various stages leading to palatal fusion. The data supports the theory that decreased cell proliferation, apparently by a disturbance at the S to M transition, might be one of the mechanism by which cleft palate is produced in the fetuses of hypervitaminosis A treated mothers.

SUMMARY

A study of the cell cycle of mesenchymal cells in the palatal processes of fetuses from hypervitaminosis A treated mothers was done. Fifty pregnant Wistar albino rats were administered 50,000 I.U. of vitamin A on days 9-11 of gestation. At day 15 of gestation they received an IP injection of 250 μCi/Kg of body weight of ^{3}HTdr. Two rats were sacrificed at 0.5 hour and hourly intervals thereafter for a period of 24 hours. Four fetus heads were recovered from each rat and prepared for autoradiographic examination. Graphs representing the percent labelled mitoses and percent labelled cells were constructed and compared to normal values. A decreased cell proliferation was found apparently as a result of a disturbance in the S to M transition.

Figure 1. Inferior-superior view of heads from rat fetuses at day 20 of gestation. The tongue has been removed. Note the fused palate of a control fetus on the upper left corner. All other fetuses are from vitamin A treated mothers. Various degrees of non-closure of secondary palate can be seen. 4X

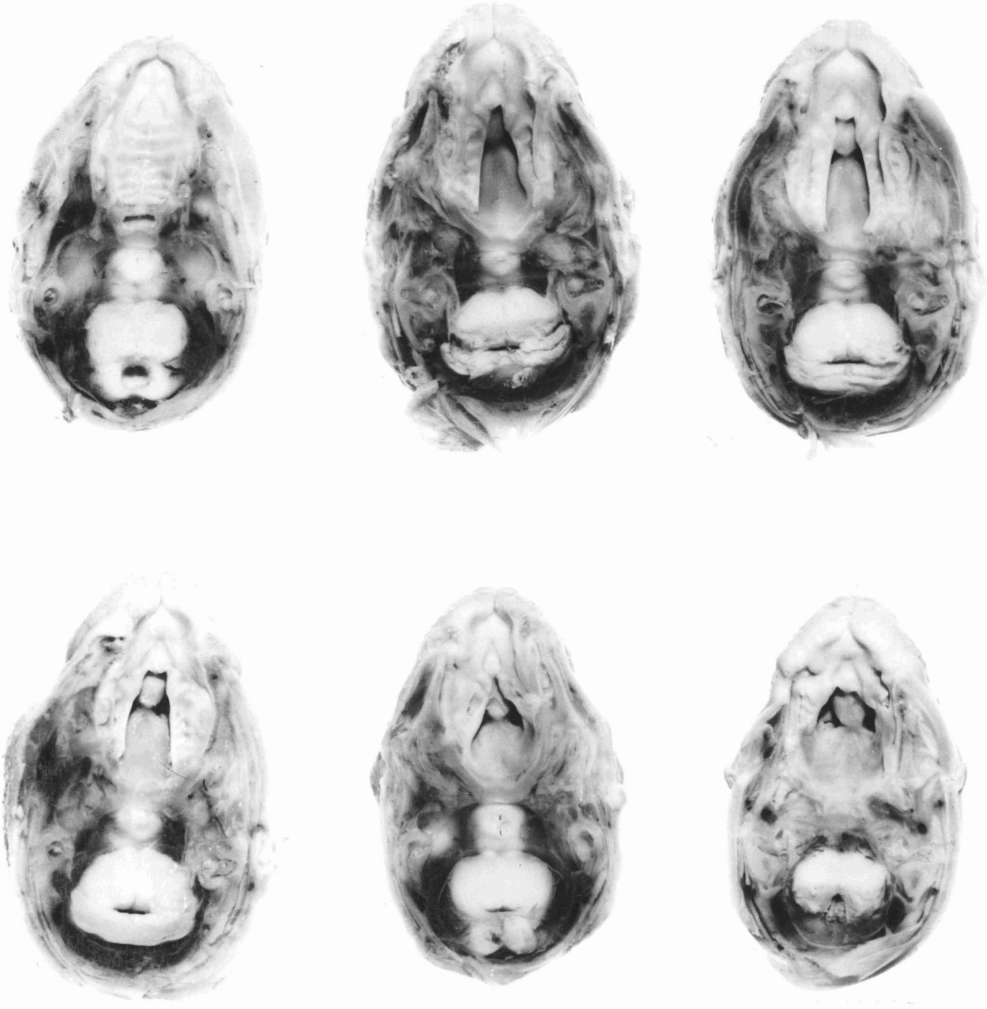


Figure 2. Frontal view of the head of a 20 day old fetus from a vitamin A treated mother. Please note the cleft of the mandible. 9X

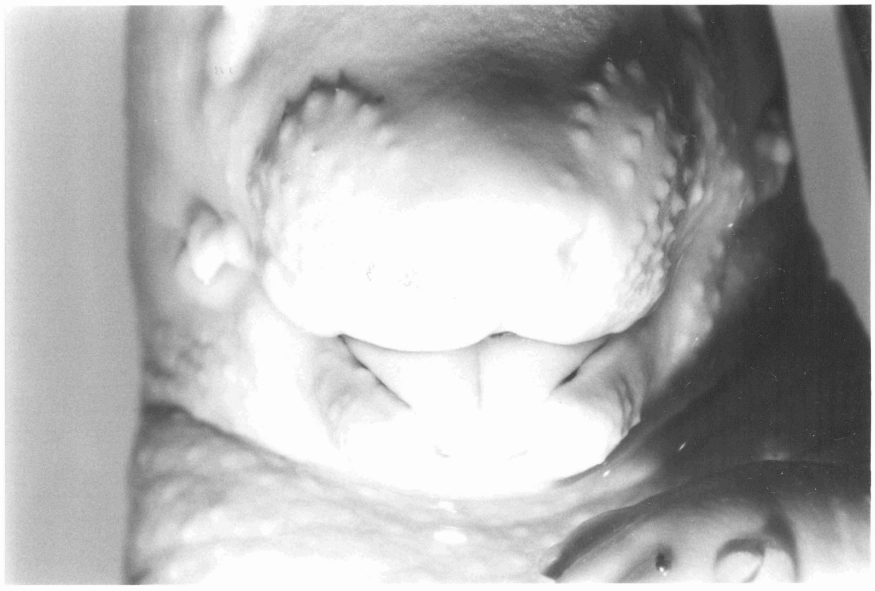


Figure 3. Frontal section of the head of a 15 day old fetus from a vitamin A treated mother. The palatal processes are vertically oriented with the tongue lying in between them. The eyes are bulbous and open. Higher magnification of the area demarcated can be seen in Figure 4. 23X

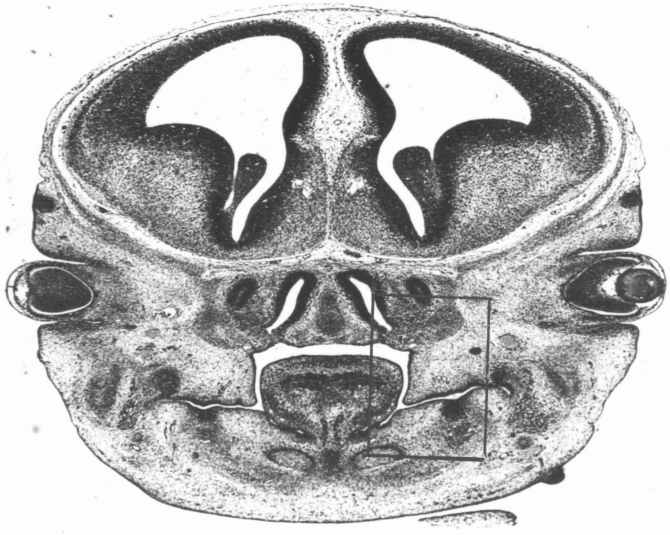


Figure 4. Higher magnification of a palatal process of the fetus shown in Figure 3. The process is composed of loose and undifferentiated mesenchymal cells covered with one or two layers of cuboidal epithelial cells. The dotted line demarcates the area utilized to count the 1000 mesenchymal cells. More than one process was necessary to count 1000 cells. 103X

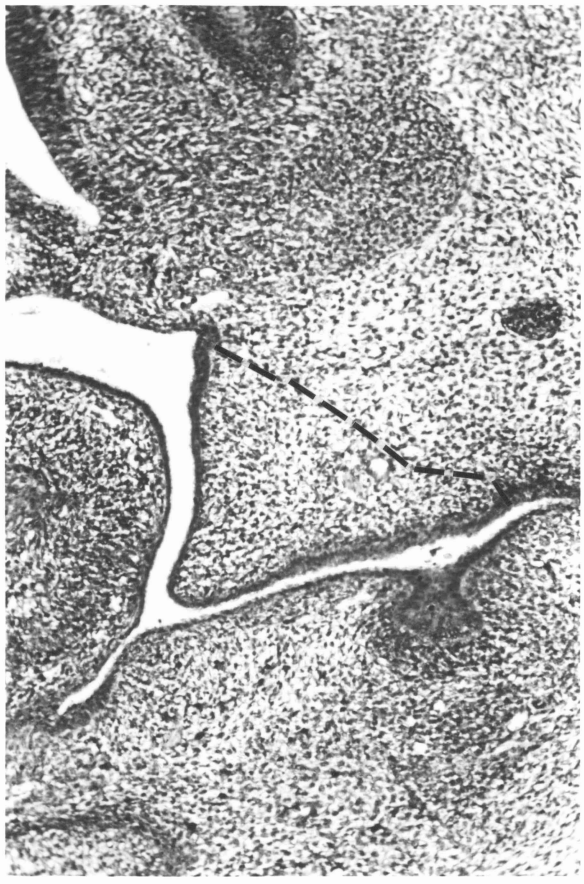


Figure 5. Higher magnification of the mesenchymal cells from the palatal process of a 15 day old fetus. Note the quality of the autoradiograph after 26 days exposure at 4°C. 1000X

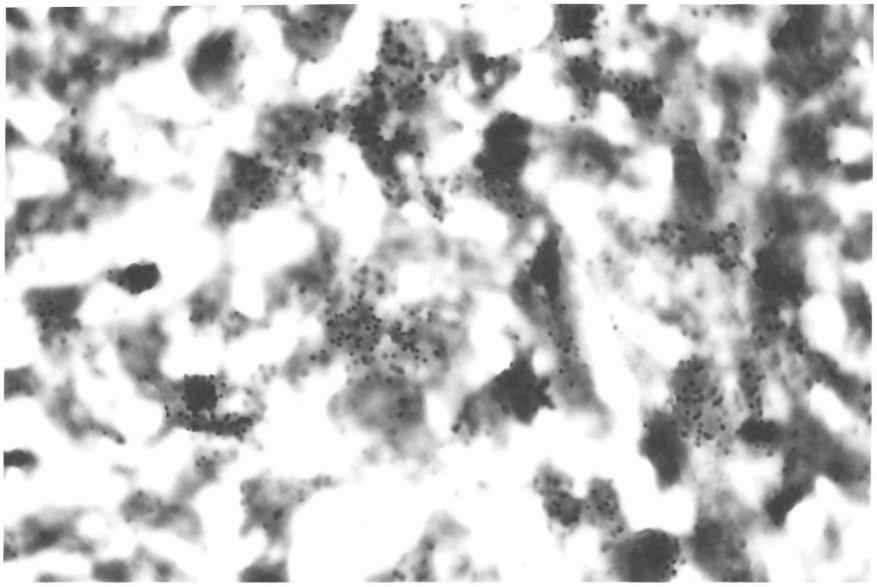


Figure 6. Graph representing the percent labelled mesenchymal cells per thousand cells counted for each hour of sacrifice.

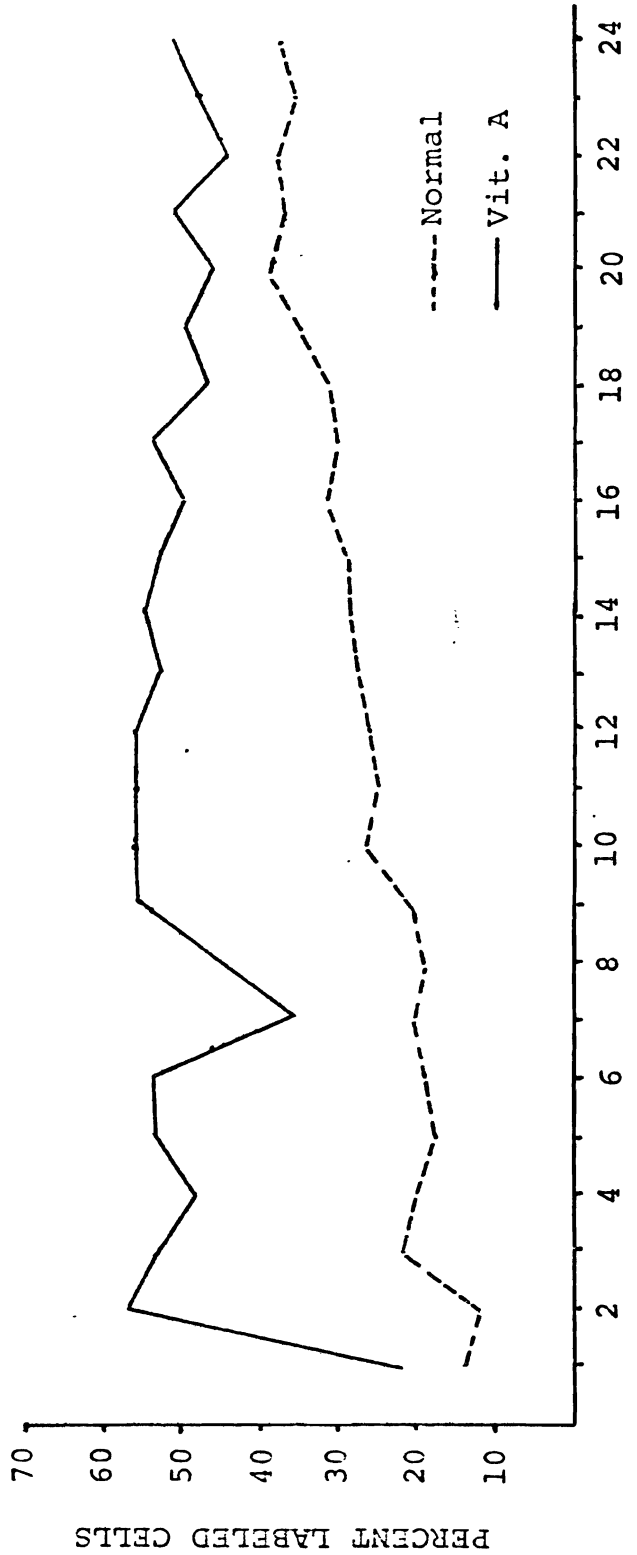
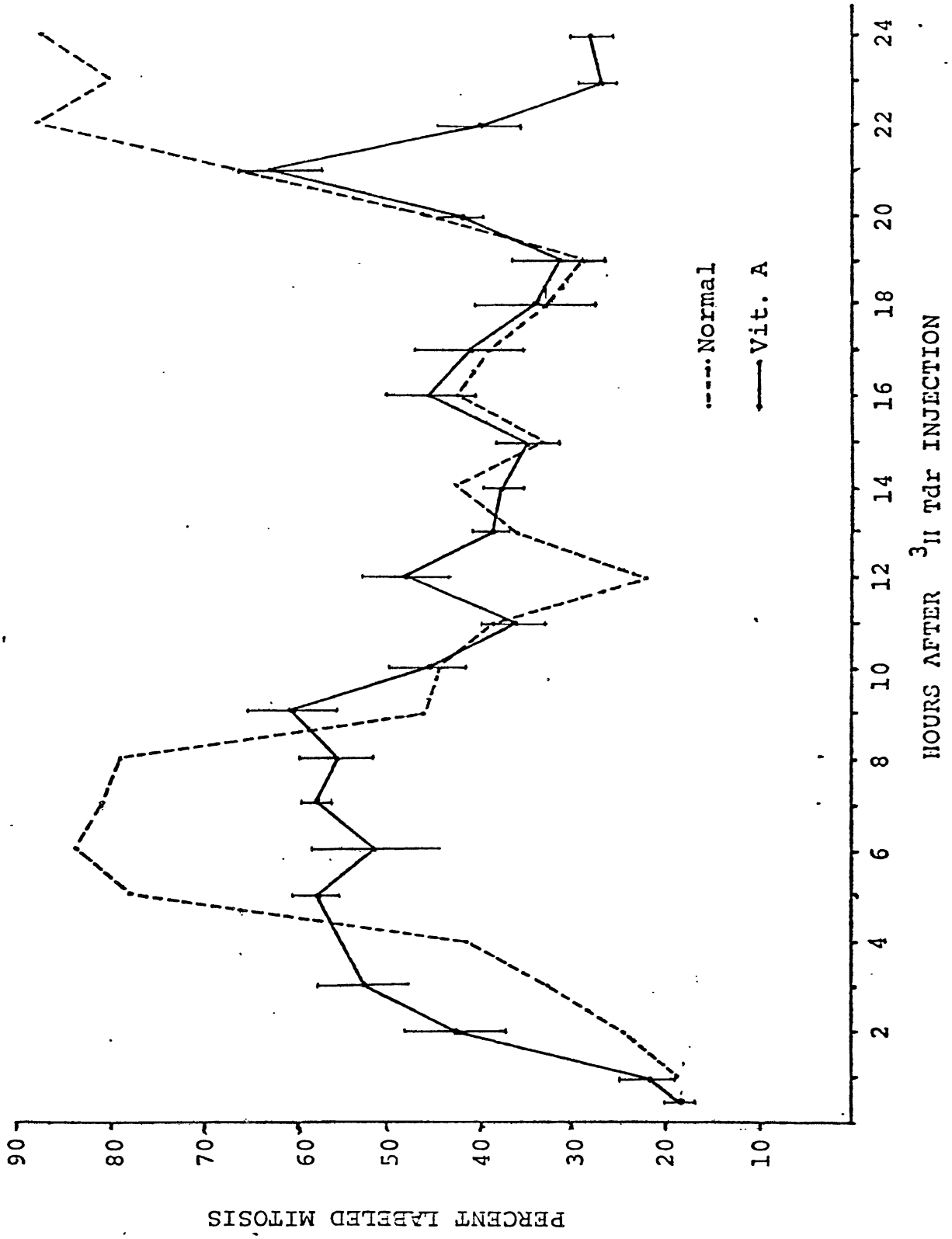
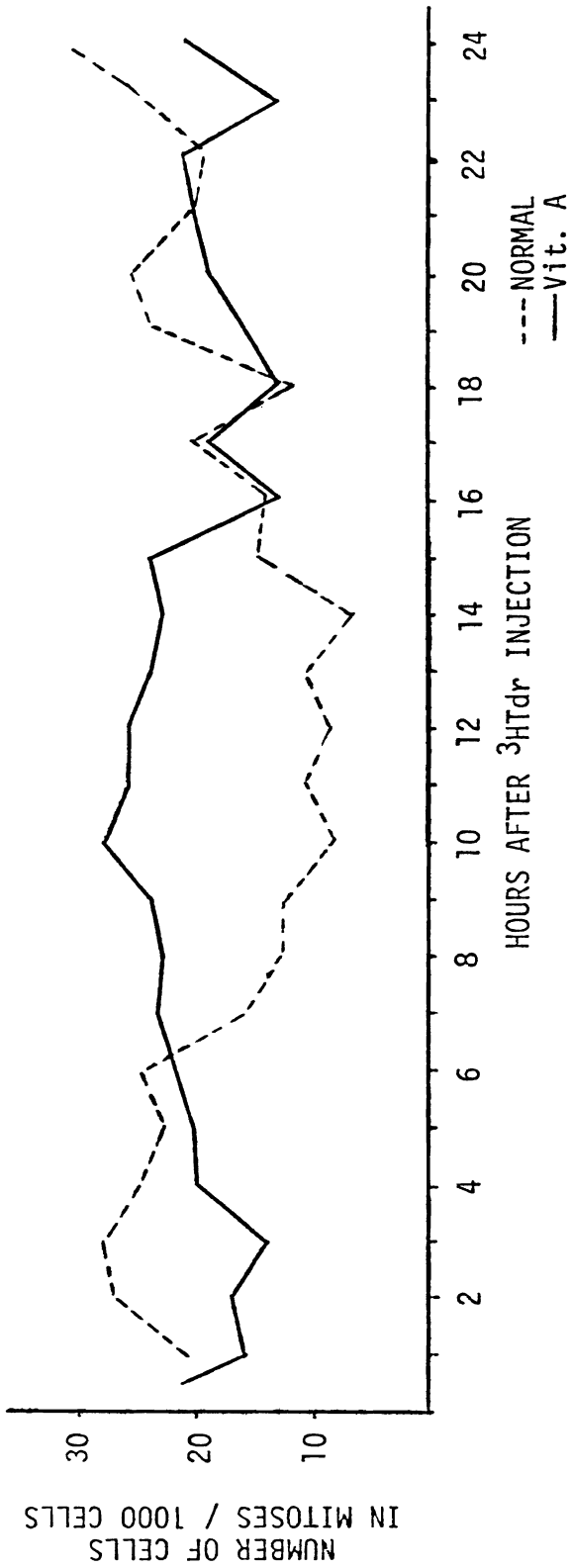

PERCENT LABELED CELLS
70
60
50
40
30
20
10
2
4
6
8
10
12
14
16
18
20
22
24
HOURS AFTER ^{3}H Tdr INJECTION
Normal
Vit. A

Figure 7. Graph representing the percent labelled mitoses per 1000 mesenchymal cells counted for each hour of sacrifice.

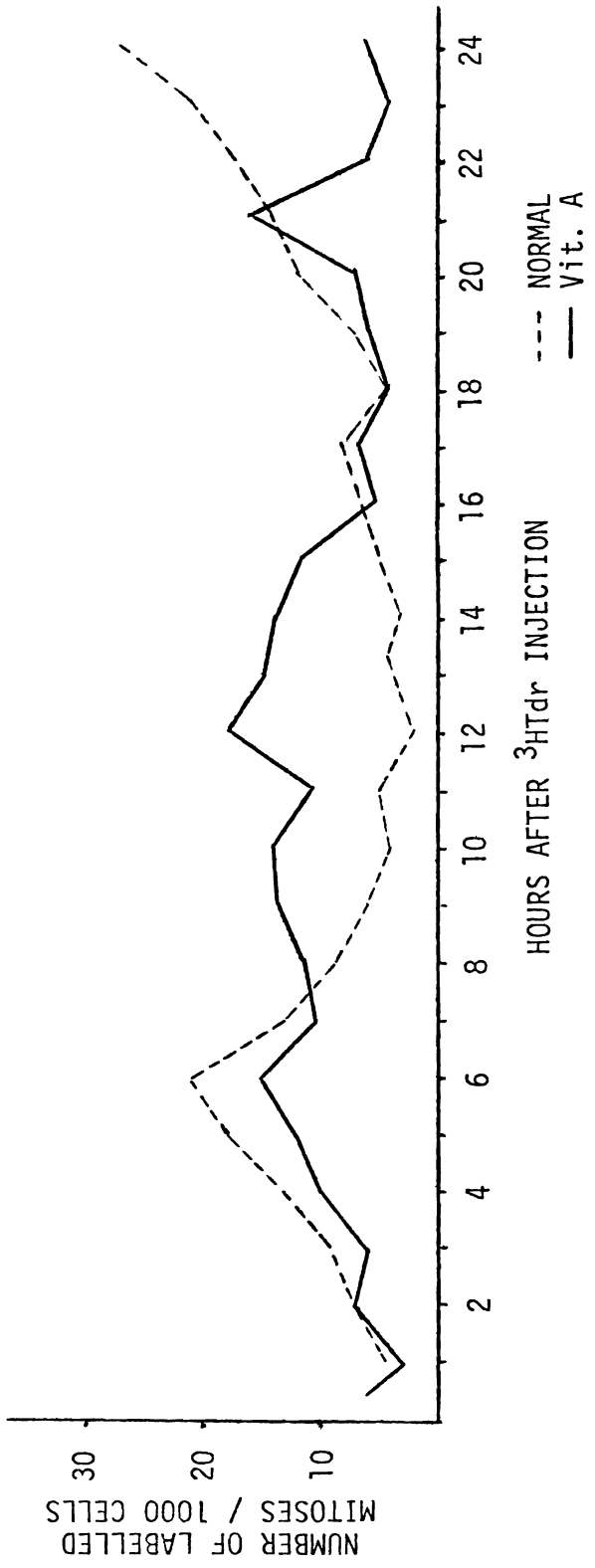

PERCENT LABELED MITOSIS
90
80
70
60
50
40
30
20
10
2
4
6
8
10
12
14
16
18
20
22
24
HOURS AFTER $^{3}_{11}$ Tdr INJECTION
Normal
Vit. A

APPENDIX

Appendix 1. Graph representing the number of cells in mitoses per 1000 cells counted for each hour of sacrifice.


NUMBER OF CELLS
IN MITOSES / 1000 CELLS
30
20
10
2
4
6
8
10
12
14
16
18
20
22
24
HOURS AFTER ^{3}HTdr INJECTION
---NORMAL
—Vit. A

Appendix 2. Graph representing the number of labelled mitoses per 1000 cells counted for each hour of sacrifice.


NUMBER OF LABELLED
MITOSES / 1000 CELLS
30
20
10
2
4
6
8
10
12
14
16
18
20
22
24
HOURS AFTER ^{3}HTdr INJECTION
--- NORMAL
— Vit. A

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